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Identification of novel genetic markers and evaluation of genetic structure in the Japanese population of the Japanese crested ibis

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Running head: GENETIC MARKER OF JAPANESE CRESTED IBIS

ABSTRACT

Japanese population of the Japanese crested ibis *Nipponia nippon* was founded by 5 individuals gifted from the People's Republic of China. In order to exactly evaluate genetic structure, we first performed development of novel genetic makers using 89 microsatellite primer pairs of related species for cross-amplification. Of them, only 3 primer pairs were useful for the genetic markers. Additionally, we sequenced allelic PCR products of these 3 markers together with 10 markers identified previously. Most markers showed typical microsatellite repeat units, but 2 markers were not simple microsatellites. Moreover, over half of the markers did not have the same repeat units as those of the original species. These results suggested that development of novel genetic markers in this population by cross-amplification is not efficient, partly because of low genetic diversity. Furthermore, the cluster analysis by STRUCTURE program using 17 markers showed that the 5 founders were divided into 2 clusters. However, the genetic relationships among the founders indicated by the clustering seemed to be questionable, because the analysis relied largely on a small number of triallelic markers, in spite of addition of the 3 useful markers. Therefore, more efficient methods for identifying large numbers of single nucleotide polymorphisms are desirable.

Key words: cross-amplification, genetic diversity, microsatellite, *Nipponia nippon*

1 INTRODUCTION

2 The Japanese crested ibis *Nipponia nippon* was a popular bird in East Asia until
3 the early 20th century, but nowadays, it is considered to be an endangered species due to
4 overhunting or reduction in available habitats (IUCN 2012). In Japan, the Japanese
5 crested ibis in the wild was extinct. After the extinction, 5 individuals (2 individuals in
6 1999, 1 individual in 2000, and 2 individuals in 2007) were introduced from **the**
7 **People's Republic of China**. As a result of the conservation efforts for captive-breeding
8 programs implemented at the Sado Japanese Crested Ibis Conservation Center, Niigata,
9 Japan, the number of the captive individuals at present are about 180 and about 90
10 individuals have been released in the Sado island.

11 In the case of small captive-breeding populations for the conservation of the
12 endangered species, it is important not only to increase the population size, but also to
13 prevent increase in inbreeding coefficient and loss of genetic diversity which cause
14 diminution of future adaptability to changing environment and reduction in the average
15 individual fitness (Lande 1988). Moreover, inbreeding depression results in reduction in
16 population growth rate and increase in extinction risk (Frankham & Ralls 1998).
17 Therefore, the exact knowledge of the current genetic diversity and structure can lead to
18 prevention of genetic deterioration, and be vital to the administration of captive
19 populations and fundamental to the success of metapopulation management strategies,
20 including the reintroduction of captive-bred individuals into the wild.

21 Historically, pedigree information has been utilized to understand the genetic
22 diversity and structure in captive populations (Ballou & Foose 1995). However, Henkel
23 *et al.* (2012) has recently reported that pedigree knowledge alone is not sufficient for the
24 exact understanding of the genetic diversity and structure, under the situation that the
25 founders of the captive population are related to each other. The Japanese population of
26 the Japanese crested ibis is applicable to this situation. Therefore, molecular genetic as

well as pedigree information are needed to assess the genetic diversity or infer the structure of the population.

Among various molecular markers, microsatellite DNA, which is simple repetitive sequences present widely throughout eukaryote organisms and have high levels of polymorphism (Jarne & Lagoda 1996; Zhang & Hewitt 2003), is one of the most useful DNA markers for the study on the genetic diversity and structure (Zhang & Hewitt 2003). For the Japanese crested ibis, 24 microsatellite markers were developed (Ji *et al.* 2004; He *et al.* 2006), and 9 of these markers were polymorphic in the Japanese population (Urano *et al.* 2013). Microsatellite PCR primer pairs derived from one species are often found to amplify microsatellite loci in related species (Engel *et al.* 1996; Primmer *et al.* 1996; Slate *et al.* 1998). Especially, cross-amplifying markers might be worthwhile for bird studies, since the avian genome has been shown to contain fewer microsatellites than organisms of other classifications (Primmer *et al.* 1997). We previously showed the polymorphisms of 10 markers developed for related species (1 from the wood stork, 1 from the roseate spoonbill, and 8 from the black-faced spoonbill) in the Japanese population and performed the assessment of genetic diversity and the inference of population structure using a total of 19 markers (Urano *et al.* 2013).

Simulation studies showed that increasing the number of loci used improved the inference of the population structure (Evanno *et al.* 2005; Hubisz *et al.* 2009). Thus, in this study, **in order to exactly evaluate genetic structure**, we **first** attempted to identify new genetic markers useful for the Japanese population using microsatellite primers developed in the related species. Subsequently, we used the whole of useful markers identified in the present and previous studies to assess the genetic diversity and infer the genetic structure of the Japanese population.

MATERIALS AND METHODS

Sample

We used the 5 founder birds for the Sado captive Japanese crested ibis population, and 138 descendant birds of which 77 individuals were alive in Japan and 61 were dead or returned to **the People's Republic of China** by 2011. The descendant birds had hatched from 2001 to 2009. For the founder birds, blood samples were collected from the founders at the Sado Japanese Crested Ibis Conservation Center (Niigata, Japan) and used for genomic DNA preparation. For the descendant birds, the post-hatch eggs stored at the Sado Japanese Crested Ibis Conservation Center were used. The vascularized chorioallantois membrane samples were excised from the eggs and used for genomic DNA preparation, as described by Urano *et al.* (2011). In addition to these samples, we used the liver sample of a dead individual for the examination of PCR amplification. DNA was extracted from these samples using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany).

PCR amplification and length polymorphism detection

We used 42, 8, 12, 1, 7, 9, and 10 microsatellite primer pairs developed for the humboldt penguin (*Spheniscus humboldti*) (Ohta A, 2010, unpublished data), the little penguin (*Eudyptula minor*) (Billing *et al.* 2007), the yellow-eyed penguin (*Megadyptes antipodes*) (Boessenkool *et al.* 2008), the african penguin (*Spheniscus demersu*) (Akst *et al.* 2002, **we used only B3-2 marker for the african penguin**), the white stork (*Ciconia ciconia*) (Shephard *et al.* 2009), the american white pelican (*Pelecanus erythrorhynchos*) (Hickman *et al.* 2008), and the great white pelican (*Pelecanus onocrotalus*) (De Ponte Machado *et al.* 2009), respectively. **These bird species were considered as the related species to the Japanese crested ibis, according to**

1 **Sibley-Ahlquist taxonomy of birds (Sibley & Ahlquist 1990). The information on**
2 **these primer pairs were also obtained from the literatures published up to 2009. All**
3 forward primers included the universal sequence (TGTAACGACGGCCAGT) at 5'
4 end. Firstly, we examined PCR amplifications of these primer pairs with DNA of a dead
5 individual. PCR reactions were performed using a 20 μ L final volume containing 20 ng
6 genomic DNA, 0.4 μ mol/L each of primers, 0.2 mmol/L each of dNTPs, 1 \times *Ex taq*
7 buffer (Takara, Shiga, Japan), and 0.5 U *Ex Taq* DNA polymerase (Takara) with the
8 following temperature profile: an initial denaturation step at 94°C for 5 min, and then 35
9 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C
10 for 1 min. PCR products were run on a 2% agarose gel and we selected the primer pairs
11 which showed a single band with a length less than 500 bp. Secondly, to detect length
12 polymorphisms in PCR products from the primer pairs, DNA panel of the 5 founders
13 were tested using 2 rounds of PCR amplification. The first-round amplifications were
14 carried out under the same condition as above. We performed second-round
15 amplifications using the first-round PCR products as the template DNA and universal
16 sequence fluorescently end-labeled with 6-FAM as the forward primer. PCR reactions
17 were performed using a 20 μ L final volume containing 1 μ L PCR products, 0.4 μ mol/L
18 each of primers, 0.2 mmol/L each of dNTPs, 1 \times *Ex taq* buffer (Takara) and 0.5 U *Ex*
19 *Taq* DNA polymerase (Takara) with the following temperature profile: an initial
20 denaturation step at 94°C for 5 min, and 30 cycles of denaturing at 94°C for 30 s,
21 annealing at 53°C for 45 s, and extension at 72°C for 45 s, followed by a final extension
22 step at 72°C for 10 min. For determining sizes of the PCR products, a portion of PCR
23 products (1 μ L) was combined with 10 μ L of formamide loading dye that contained the
24 fluorescent labeled GeneScan-500LIZ size standard (Applied Biosystems, Foster city,
25 CA), and resolved on the 3730xl DNA analyzer (Applied Biosystems). We used
26 GeneMapper software version 4.0 (Applied Biosystems) to score the PCR products. The

primer pairs showing polymorphisms among the 5 founders were ascertained the effectiveness as genetic markers by comparing the genotypes of individuals with Mendelian parent-offspring relationships. Subsequently, the markers which were seemed to be effective were genotyped on the descendants.

Validation of polymorphic markers as microsatellites

We sequenced the polymorphic markers identified in this study and the 10 related species markers identified in the previous study (Urano *et al.* 2013) and examined the differences in the sequence among PCR products of different size. PCR amplifications were carried out on the same condition as in the examination of amplification, except for the temperature profile for the previously identified markers, which followed the protocols described by He *et al.* (2006), Sawyer and Benjamin (2006), Tomasulo-Seccomandi *et al.* (2003), and Yeung *et al.* (2009). The PCR products were examined by electrophoresis through a 2% agarose gel to determine the quality and quantity for DNA sequencing. DNA sequencing of PCR products was performed by direct sequencing following excision from the gel. Alternatively, the PCR products were cloned into TA cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and at least 2 positive clones were sequenced. Sequencing was carried out by Greiner Japan Co., Ltd. (Tokyo, Japan). Sequence alignments and comparisons were made with GENETYX version 10 (Software Development, Tokyo, Japan). Nucleotide polymorphisms were identified by comparison of sequence traces between the PCR products of different size.

Data analysis

Based on the whole of useful markers identified in the present and previous

studies, basic genetic diversity parameters including allelic diversity (A ; Fernández *et al.* 2005), effective number of alleles (n_e), observed heterozygosity (H_o), unbiased expected heterozygosity (H_E ; Nei 1987), and polymorphic information content (PIC; Boststein *et al.* 1980) of the Japanese population were estimated using EXCEL MICROSATELLITE TOOL KIT version 3.1.1 (Park 2001). The deviation from Hardy-Weinberg equilibrium (HWE) at each locus was tested using a Markov-chain algorithm (Guo & Thompson 1992) in GENEPOP version 4.0 (Raymond & Rousset 1995). All pairs of loci were also tested for genotypic linkage disequilibrium using the exact test in GENEPOP version 4.0. The sequential Bonferroni adjustment (Rice 1989) was applied to correct for multiple testing of deviation from HWE and linkage disequilibrium.

To investigate the genetic structure of the population, program STRUCTURE version 2.3 (Pritchard *et al.* 2000) was used. Since we found that different runs could produce different likelihood values, 10 runs were carried out for each data set in order to quantify the amount of variation of the likelihood for each K , or the number of clusters. The range of possible K s we tested was from 1 to 8. Runs with a burn-in period of 100,000 followed by 100,000 MCMC iterations were performed, using the model with correlated allele frequencies and assuming admixture (Falush *et al.* 2003). The α , or the Dirichlet parameter for the degree of admixture, was the same for all clusters and allowed to vary between runs. For each value of K , the log-likelihood values of the data defined as $\ln \Pr(X/K)$ (Pritchard *et al.* 2000) were averaged and standard deviation was calculated. We tried to infer the appropriate K by comparing both the averaged $\ln \Pr(X/K)$ and calculated ΔK statistic (Evanno *et al.* 2005). This procedure was repeated 3 times. Additionally, the same analysis was carried out, using the subsets of markers in order to investigate the influence of the marker sets used on STRUCTURE results.

RESULTS

Identification of novel useful marker

Of the 89 primer pairs (Billing *et al.* 2007; Boessenkool *et al.* 2008; Hickman *et al.* 2008; De Ponte Machado *et al.* 2009; Shephard *et al.* 2009; Ohta A, 2010, unpublished data), 25 (13 from the humboldt penguin, 3 from the american white pelican, 5 from the white stork, and 4 from the great white pelican) succeeded in amplifying (out of the other 64 primer pairs, 18 showed multiple bands, but 46 showed no PCR products). Out of the 25 primer pairs, 4 (hSTR1-78, hSTR1-92 and hSTR1-150; from the humboldt penguin, and PEL086; from the great white pelican) had PCR products with different size among the 5 founders (Table 1). Remaining 21 loci amplified as a monomorphic microsatellite product in this study: hSTR1-56, hSTR1-102, hSTR1-109, hSTR1-139, hSTR1-162, hSTR1-233, hSTR1-268, hSTR1-308, hSTR1-312, and hSTR1-344 from the humboldt penguin, PeEr03, PeEr05, and PeEr06 from the american white pelican, Cc01, Cc02, Cc05, Cc06, and Cc07 from the white stork, and PEL149, PEL207, and PEL304 from the great white pelican. We should note that all the 5 founders had the same hetero-genotypes at each of 2 primer pairs (hSTR1-78 and hSTR1-92). To ascertain whether the 4 primer pairs are useful as genetic markers, the PCR products were genotyped using DNA from individuals with Mendelian parent-offspring relationships. As a result, we obtained the genotypes consistent with Mendelian parent-offspring relationships at 3 primer pairs (hSTR1-78, hSTR1-150, and PEL086), but that was not the case at 1 primer pair (hSTR1-92) where all the individuals showed the same hetero-genotypes (data not shown). Thus, the 3 polymorphic markers were chosen for large-scale genotyping of the 138 descendants for the **exact** analysis of genetic diversity and structure in this study.

Table 1

For the 3 markers together with the 10 related species markers identified in the

previous study, we sequenced allelic PCR products of different size. Of the 13 markers, 11 had repeat regions and there were differences in the number of repeat unit among each allele, while 2 markers did not seem to be simple microsatellite markers (Table 2, Fig. 1). There were three and one partial differences in sequences between alleles of PM1-17 and PM2-37 loci, respectively, and the variations of single nucleotide were considered to be sequencing errors or single nucleotide polymorphisms. For the 11 markers which had simple repeat regions, comparing the sequences of repeat motifs with those of original species, we found that 6 had the same repeat motifs as those of the original species, whereas 5 had different sequences from the original species.

Table 2

Analysis of genetic diversity

Fig. 1

The genetic diversity parameters were shown in Table 3. The values of the first 19 markers were cited from Urano *et al.* (2013), but slightly different values were obtained because we recalculated them excluding some data which were inconsistent with Menderian parent-offspring relationships probably because of typing error. The 3 useful markers identified in this study had 7 alleles in total (3 alleles at PEL086 locus and 2 alleles each at hSTR1-78 and hSTR1-150 loci). For the whole of 22 useful markers (3 identified here and 19 identified previously), average A was 2.18 in both the founders and descendants and average n_e , H_E , H_O , and PIC were 1.73, 0.38, 0.40, and 0.31, respectively. PEL086 locus had the second highest values for these parameters, and hSTR1-78 and hSTR1-150 loci had moderate values. The 77 descendants which are still alive in Japan inherited all alleles of 22 markers from the founders and the genetic diversity measures similar to those of the 138 descendants were obtained (data not shown). The Nn26, NnNF5, and PM2-20 loci and the overall showed a deviation from the HWE ($P < 0.05$) and the NnNF5 locus significantly deviated from HWE even after

sequential Bonferroni correction for multiple testing. The PM2-20 locus may be Z-linked since the locus had H_o lower than expected, and in the original species, or black-faced spoonbill, it was suggested that the marker was Z-linked (Yeung *et al.* 2009). In addition, pairwise tests for genotypic disequilibrium revealed that all the 3 markers identified in this study (hSTR1-150, hSTR1-78, and PEL086) were in significant linkage disequilibrium with previous markers (Nn01, PM2-28, and Aaju02) after accounting for multiple testing.

Table 3

Cluster analysis

For the analyses of the composition of genomes among the founders and the descendants using the program STRUCTURE, we used 17 markers except for the PM2-20 locus which seemed to be Z-linked, NnNF5 locus which showed a significant deviation from HWE even after sequential Bonferroni correction, and hSTR1-150, hSTR1-78, and Aaju02 for the 3 pairs of loci which were in significant linkage disequilibrium. In the program STRUCTURE, the true number of clusters (K) is often identified using the maximal value of $\ln \Pr(X/K)$ (Ciofi *et al.* 2002; Vernesi *et al.* 2003; Hampton *et al.* 2004). The highest value of $\ln \Pr(X/K)$ was observed at $K = 2$ for all 3 replicates. Furthermore, the ΔK statistic was found to be also obviously maximum at $K = 2$, giving averaged ΔK values for 3 replicates of 89.28 at $K = 2$, 2.01 at $K = 3$, 3.79 at $K = 4$, 2.66 at $K = 5$, 2.68 at $K = 6$, and 5.56 at $K = 7$. This finding suggested that the Japanese population contains 2 clusters. At $K = 2$, as shown in Fig. 2, the 2 clusters were distributed with approximately equal proportion in total and with considerable inter-individual variation, suggesting that the founder genomes were inherited equally in the population and with various admixture patterns at individual level. Given that this population was founded by 5 individuals, the result of only 2 clusters was likely to be

Fig. 2

consistent with a considerably low level of genetic diversity in the Japanese population.

We carried out the cluster analysis with the subsets of the 17 markers. The result obtained by excluding the 4 triallelic markers (Nn01, PM2-21, PM2-37, and PM3-13) from the 17 markers was largely different from that of the 17 markers (Fig. 3), giving the highest value of $\ln \Pr(X/K)$ at $K = 1$. However, inclusion of the 4 triallelic markers, irrespective of the markers chosen, resulted in the result similar to that of the 17 markers. Even if we used the 4 triallelic markers in combination with the 6 biallelic markers which had low PIC values (Nn17, Nn18, PM2-16, PM2-37, PM3-13, and Wsu13), we obtained the similar result at $K = 2$ (data not shown). Therefore, it was suggested that the triallelic markers had an essential effect on the clustering result.

Fig. 3

Genetic relationships among the founders

The analysis by STRUCTURE showed that 5 founders were divided into 2 clusters. One cluster contained founder C which was accounted for mostly by dark gray cluster and another cluster contained founder A, B, D, and E that was accounted for mostly by light gray cluster. Furthermore, founder C was also distinct from the other founders at $K = 3$ to 7. However, the genotype data did not show that there was an apparent difference between the 2 clusters (Table 3). The founder C had a specific allele for Nn01 and specific genotypes for PM221, hSTR1-150, and PEL086, but over 80% of the genotypes of founder C were shared by the other founders. Therefore, the genome of founder C did not seem to be apparently distinct from the others' genomes.

DISCUSSION

Because the isolation of microsatellite markers is extensive and time-consuming, it is generally efficient to test the cross-amplification of the primer of

the related species (Galbusera *et al.* 2000). In this study, of the 89 microsatellite markers developed for the related species, 25 were successfully amplified, 4 showed PCR products of different size in the 5 founders, and eventually, **only 3** were **indicated** to be useful as a genetic marker for the Japanese population. In the previous study, 44 out of the 50 markers developed for the related species were successfully amplified and 10 showed polymorphisms in the 5 founders (Urano *et al.* 2013). The proportion of the markers which were successfully amplified or showed polymorphisms in this study was extremely low, as compared with that of the previous study, being consistent with the fact that the related species utilized in this study were evolutionally more distant from the Japanese crested ibis than those of the previous study. The species utilized in this study belong to different families from the Japanese crested ibis, while the most of the species used in the previous study belong to the same family as the Japanese crested ibis (Sibley & Ahlquist 1990; Urano *et al.* 2013). It is suggested that the evolutionally more distant the related species used are from the target species, the lower the probability of amplification success or polymorphism detection is (Primmer *et al.* 1996, 2005). Particularly, in bird species, it was reported that in the case where the related species used belonged to a different family **of the same order**, the rate of success in amplification (**33 ± 3%**) or detection of polymorphisms (**28 ± 6%**) was lower than that of the case where they belonged to **a different genus of the same family (60 ± 2% or 43 ± 3%)** (Primmer *et al.* 2005; Barbará *et al.* 2007). We should note that it is also difficult to identify new markers in a population with lower genetic diversity such as the population of the Japanese crested ibis.

It is necessary to determine the sequence of alleles in order to validate the genetic markers from the cross-amplification as microsatellite, because it is unclear that the primers amplify the microsatellite sequences in the target species. Thus, we sequenced the alleles of the 3 markers identified in this study and the 10 markers

identified previously by the cross-amplification (Urano *et al.* 2013). As a result, we found that while 11 markers have tandem repeat sequences, 5 markers have the repeat sequences different from those of the original species. Also, 2 markers were not simple microsatellite. These results suggested that over half of the markers did not amplify orthologous genome regions to the original species.

Using the whole of useful markers identified in the present and previous studies, we calculated genetic diversity parameters in the Japanese population. Although the 3 markers were added and one of those was triallelic, values of the parameters were not much different from the previous study by Urano *et al.* (2013) and were similar to those of other species with population bottleneck (Bouzat *et al.* 1998; Tarr *et al.* 1998). These results, together with the result that the maximum number of alleles at single locus was 3 in spite of the founding by 5 individuals, support our previous idea of a considerably low level of genetic diversity in the Japanese population (Urano *et al.* 2013).

In the result of the cluster analysis by STRUCTURE, the 5 founders were divided into 2 clusters. On the other hand, comparing the actual genotype data, there was not apparent difference between 2 clusters. Furthermore, by excluding the 4 triallelic markers, the founders were not divided into 2 clusters. These results suggest that the cluster analysis relied largely on more polymorphic markers and the differences in the genotypes of the triallelic markers made a significant contribution to the STRUCTURE result. Thus, the genetic relationships among the 5 founders indicated by the cluster analysis with STRUCTURE using 17 markers seemed to be questionable, because the analysis relied largely on a small number of triallelic markers, **in spite of addition of the 3 useful markers**. Therefore, development of large numbers of **single nucleotide polymorphisms** by means of more efficient methods such as those using next generation sequencing technologies and further molecular genetic studies with those markers will be needed for more exact understanding of genetic diversity and

1 structure in the Japanese population of the Japanese crested ibis. Additionally, for the
2 future conservation management strategy, it is important to investigate not only neutral
3 polymorphisms in microsatellite markers but also functional mutations related to fitness
4 in MHC (Major histocompatibility complex) regions (Kohn *et al.* 2006; Agudo *et al.*
5 2011).

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Figure Legends

Fig. 1 Nucleotide sequence alignment of the two PCR products by primers PM1-17 (a) and PM2-37 (b). The upper and lower rows show the sequence of the one and the other PCR product, respectively.

Fig. 2 Genetic structure of the Japanese population in Japan consisting of the 5 founders and 138 descendants based on the STRUCTURE software with 17 markers. Each animal is represented by a single vertical line divided into $K = 2$ colors, where K is the number of clusters assumed and the length of the colored segment represents the individual's estimated proportion of membership to a particular cluster. The characters A to E indicate the 5 founders. Among the 10 runs carried out for $K = 2$, only the graphical representation of the highest estimates for $\ln \Pr(X/K)$ value is given.

Fig. 3 STRUCTURE result obtained with 13 markers, removing 4 triallelic markers from the whole 17 markers. Each animal is represented by a single vertical line divided into $K = 2$ colors and the length of the colored segment represents the individual's estimated proportion of membership to a particular cluster. The characters A to E indicate the 5 founders.

Fig.1(b)

```
CATACAAGCAATGCCTAACCATACCTCTGAGTTTCATGTATGTAGATAGATTTCCCTTCTCTTAGAAATGGAGGTTAAGTGTTGTCCAAA
|||||
CATACAAGCAATGCCTAACCATACCTCTGAGTTTCATGTATGTAGATAGATTTCCCTTCTCTTAGAAATGGAGGTTAAGTGTTGTCCAAA

TCAATACTGATTCAATTGTACTATTCAAAGATTTCTCACTGTTATGAATTTAGGGTATTTTTGAAAAAGAAAGAAAGAAGGAAAGAGAG
|||||
TCAATACTGATTCAATTGTACTATTCAAAGATTTCTCACTGTTATGAATTTAGGGTATTTTTGAAAAAGAAAGAAAGAAGGAAAGAGAG

AGAAAGAAAGAAAGAACGAAAGAACGAAAGAAC-----AAAAGAAAGAAAGAATGAAAG
|||||
AGAAAGAAAGAAAGAACGAAAGAACGAAAGAACGAAAGAAAGAATGAAAGAAAGAACGAAAGAAAGAACAAAAGAAAGAAAGAATGGAAG

AACAAAAGAAAGAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAA
|||||
AACAAAAGAAAGAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAA

AAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAA
|||||
AAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAA

GAAAAAGAAAAAGAAAAAGAAAAAGTTGTGTAGTTTCAGTCTGAGT
|||||
GAAAAAGAAAAAGAAAAAGAAAAAGGTTGTGTAGTTTCAGTCTGAGT
```


Fig.2

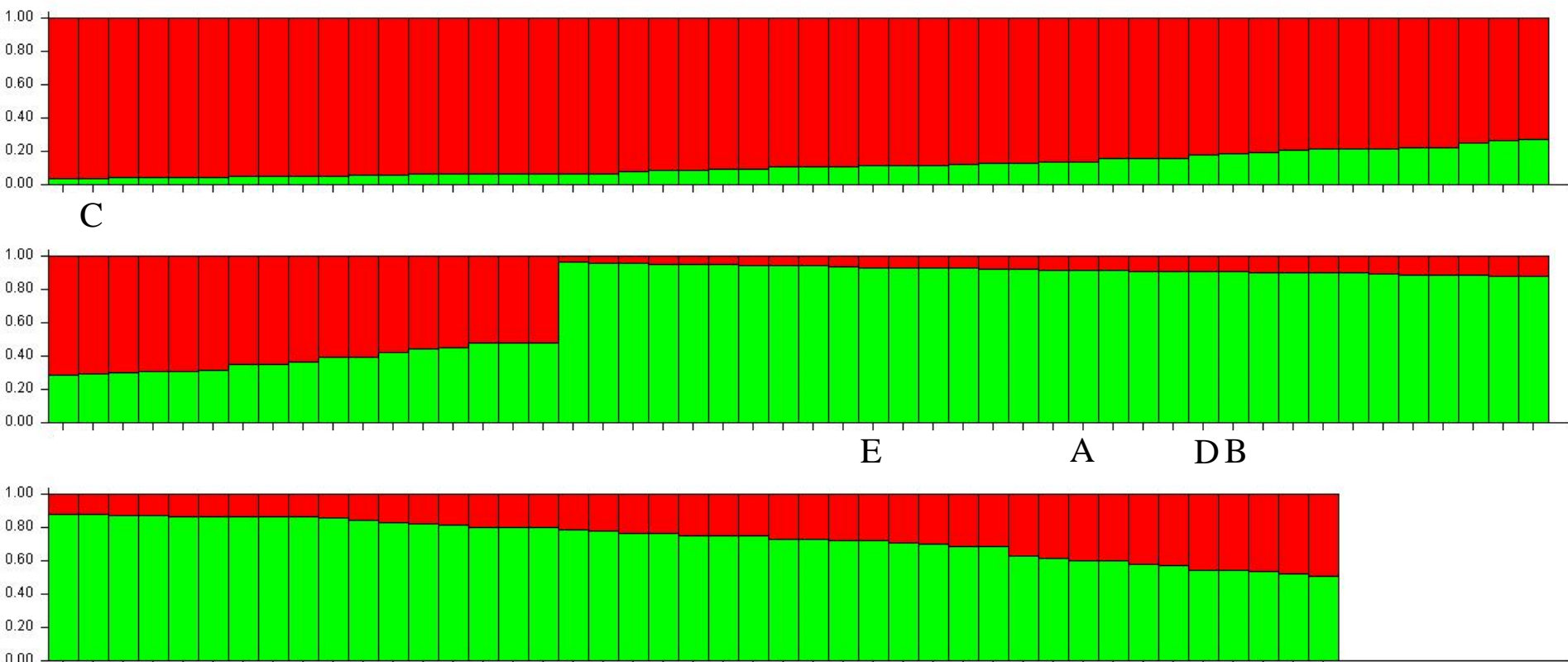


Fig.3

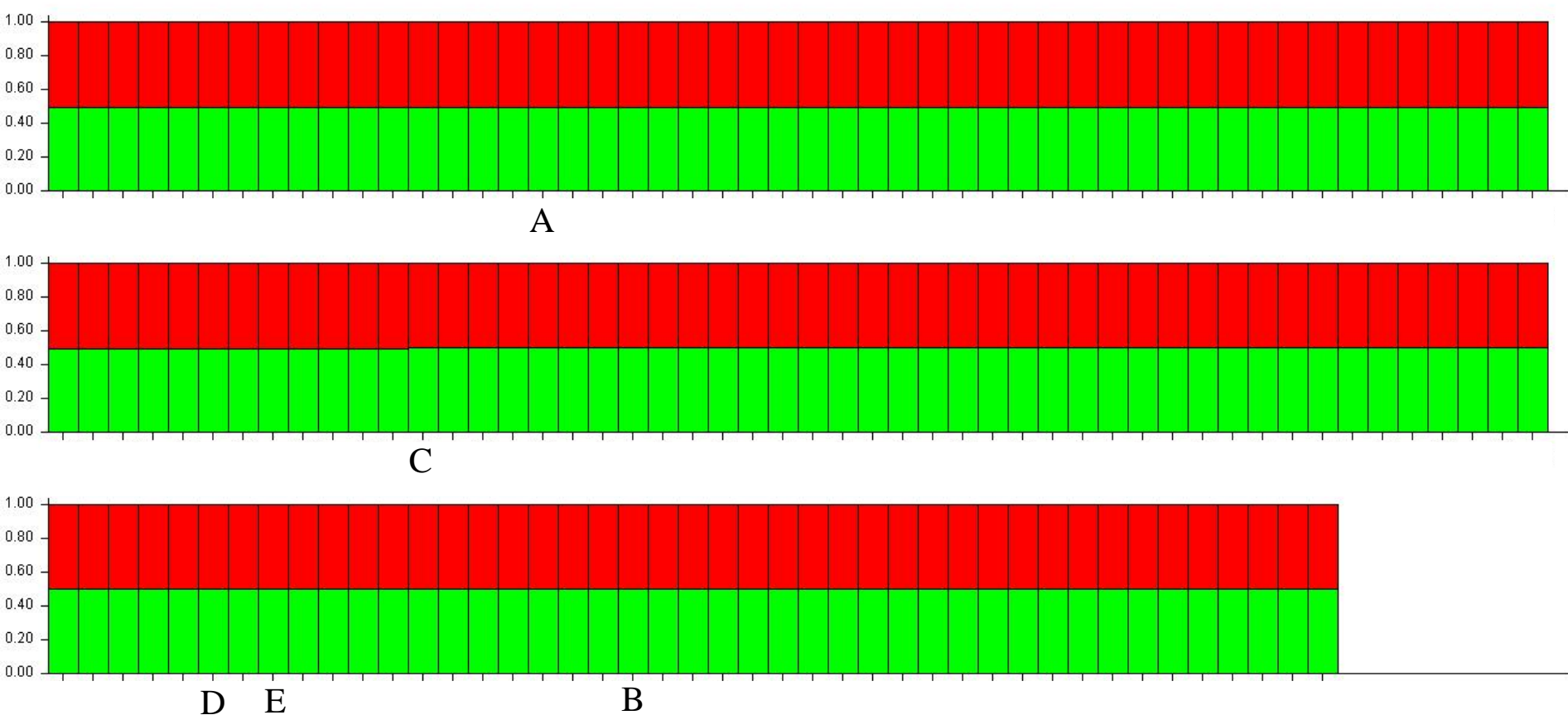


Table 1 The size of PCR products for 5 founders

Locus	Source species	Size of PCR products for each founder [†] (bp)					Reference
		A	B	C	D	E	
hSTR1-78	Humboldt penguin	267, 271	267, 271	267, 271	267, 271	267, 271	Ohta A, unpublished data
hSTR1-92	Humboldt penguin	111, 115	111, 115	111, 115	111, 115	111, 115	Ohta A, unpublished data
hSTR1-150	Humboldt penguin	320, 325	320, 325	325	320, 325	320, 325	Ohta A, unpublished data
PEL086	Great white pelican	189, 193	185, 193	185, 189	189, 193	189	De Ponte Machado et al. (2009)

[†]The characters A to E represent founders.

Table 2 The repeat motif and the number of repeat unit of 2 or 3 alleles for each loci

Locus	Repeat motif of original species	Repeat motif [†]	Number of repeat motif [†]
PM1-17	AAAG	-	-
PM2-16	AAAG	(TTTA) _n	n=6 or 7
PM2-20	AAAG	(AAAG) _n	n=12 or 13
PM2-21	AAAG	(GGAAGAAA) _n	n=12, 14 or 17
PM2-28	AAAG	(AAAG) _n	n=6 or 7
PM2-29	AAAG	(AAAG) _n	n=13 or 14
PM2-37	AAAG	-	-
PM3-13	CA	(CA) _n	n=5 or 6
Aaju02	(AACT) ₁₃ TA(CTTT) ₆	(CTTTT) _m CTTTT	m=20 and n=2 or
	CT(CTTT) ₃	(CTTTT) _n	m=23 and n=1
Wsu13	AC	(AC) _n	n=13 or 14
hSTR1-78	AC	(CAAA) _n	n=3 or 4
hSTR1-150	TATCC	(AGAAC) _n	n=4 or 5
PEL086	TATC	(TATC) _n	n=8, 9 or 10

[†]The characters “m” and “n” represent the number of repeat unit.

Table 3 Allelic diversity (A), genotypes of the 5 individuals, effective number of alleles (n_e), unbiased expected heterozygosity (H_E), observed (direct count) heterozygosity (H_O), polymorphic information content (PIC) and deviation from HWE in the Japanese population (the founders and descendants) of 22 microsatellite markers

Locus	Allelic diversity	Genotype of founder [†]					n_e	H_E	H_O	PIC	HWE [‡]
		A	B	C	D	E					
Nn01	3	12	12	13*	12	11	2.01	0.50	0.53	0.45	
Nn04	2	12	22	12	12	12	1.90	0.47	0.54	0.36	
Nn12	3	13	23	23	12	13	2.98	0.67	0.72	0.59	
Nn17	2	22	22	22	22	1*2	1.02	0.02	0.02	0.02	
Nn18	2	22	12	12	22	12	1.43	0.30	0.33	0.26	
Nn21	2	12	11	12	22	12	1.99	0.50	0.58	0.37	
Nn25	2	12	12	22	22	12	1.87	0.47	0.43	0.36	
Nn26	2	11	12	12	12	12	1.63	0.39	0.46	0.31	*
NnNF5	2	12	22	12	22	12	1.57	0.37	0.48	0.30	*, (*)
PM1-17	2	11	12	12	12	12	1.91	0.48	0.51	0.36	
PM2-16	2	11	12	11	22	22	1.36	0.26	0.28	0.23	
PM2-20	2	11	12	11	12	11	1.25	0.20	0.16	0.18	*
PM2-21	3	13	12	11	12	13	2.39	0.58	0.58	0.50	
PM2-28	2	11	12	12	12	12	1.69	0.41	0.46	0.32	
PM2-29	2	12	22	12	22	22	1.80	0.45	0.42	0.35	
PM2-37	2	22	22	22	1*2	22	1.01	0.01	0.01	0.01	
PM3-13	2	1*2	22	22	22	22	1.21	0.17	0.19	0.16	
Aaju02	2	22	12	12	22	12	1.80	0.45	0.40	0.35	
Wsu13	2	11	11	12	12	11	1.31	0.24	0.28	0.21	
hSTR1-78	2	12	12	12	12	12	1.66	0.40	0.44	0.32	
hSTR1-150	2	12	12	22	12	12	1.49	0.33	0.33	0.28	
PEL086	3	13	23	12	23	22	2.83	0.65	0.67	0.57	
Mean	2.18						1.73	0.38	0.40	0.31	*

[†]The number 1, 2, 3 represents each allele, the allele specifically carried by one founder is indicated by asterisk, and the genotype specifically carried is indicated in bold.

[‡]Significant deviation from Hardy-Weinberg equilibrium is indicated by asterisk ($P < 0.05$), and that after correction using Bonferroni procedure is indicated by asterisk in bracket ($P < 0.05/22=0.002$).